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Broad spectrum of *in vivo* forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: Substitutions, frameshifts, and hypermutations*

(*lac* repressor/DNA purification)

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ABSTRACT **We determined the *in vivo* forward mutation rate in a single replication cycle for spleen necrosis virus (SNV). A method was developed to clone integrated proviruses of retroviral shuttle vectors by exploiting the tight binding of the *lac* operator to the *lac* repressor protein. The vectors contained the *lacZα* gene as a reporter of mutations. Thirty-seven of the 16,867 proviruses recovered contained five classes of mutations, including substitutions and frameshifts. Runs of 9 and 10 identical base pairs and a direct repeat of 110 base pairs were mutational hotspots. In addition, two copies of a provirus contained 15 G-to-A substitutions. Such proviruses, which we name hypermutants, may arise through the action of an error-prone polymerase and could significantly contribute to the genetic variation in retroviral populations.**

Variation within retrovirus populations has been observed since their discovery (1). It was later noted that chicken embryo fibroblast transformants with distinct focus morphologies produce viruses that upon passage produce foci of the same characteristic morphologies. These viruses frequently mutated to produce variant foci of different focus morphology (2, 3). The rates at which the retroviral v-*mos* oncogene of Moloney murine sarcoma virus and its cellular counterpart c-*mos* accumulated mutations were estimated as 1.31×10^{-3} substitution per site per year and 1.71×10^{-9} substitution per site per year, respectively (4).

Three different polymerases are involved in retrovirus DNA synthesis. Host cell DNA polymerases replicate the provirus in each cell cycle. RNA polymerase II synthesizes the RNA genome of the next generation of virions. Virus-encoded reverse transcriptase synthesizes a DNA copy of the RNA genome that integrates into the host genome as a provirus. Because at least one cellular DNA polymerase (δ) possesses an exonucleolytic proofreading mechanism, cellular DNA replication exhibits low mutation rates [10^{-9} to 10^{-11} substitution per base pair (bp) per cell division] (5), so that its contribution to the retroviral mutation rate should be negligible. However, the error rate of cellular RNA polymerase II has not been measured, and its contribution to retroviral mutation rates is unknown.

Previous *in vitro* measurements of reverse transcriptase mutation rates utilizing a *lacZα* gene assay or a mutated bacteriophage (ϕX174) assay range from 10^{-4} to 10^{-5} substitution per bp per cycle of polymerization (6–10).

Mutation rates derived *in vivo* could differ from mutation rates derived *in vitro* because alterations in fidelity could occur as a result of alteration of polymerases during purification or of cell-free conditions of DNA synthesis. Oligonucleotide fingerprinting analysis or denaturing gradient gel analysis of replication-competent Rous sarcoma virus stocks was performed, and base substitution rates were estimated to be 3×10^{-4} per passage and 1.4×10^{-4} per bp per cycle, respectively (11, 12). The *in vivo* rate of reversion of an A-to-G substitution mutation for a spleen necrosis virus (SNV)-based retroviral vector in a single cycle of replication was 2×10^{-5} substitution per bp per cycle (13).

We designed a system to determine the *in vivo* forward mutation rate for a single retrovirus replication cycle. In addition to substitutions, frameshifts, and mutational hotspots, we unexpectedly encountered two proviruses that had 15 base-pair substitutions in a 930-bp region. We call the process by which individual proviruses acquire several mutations in a single replication cycle hypermutation, and we suggest that such hypermutant proviruses arise through the action of error-prone polymerases, which themselves arise as a result of errors occurring during transcription or translation.

MATERIALS AND METHODS

Plasmid Construction. Retroviral vector plasmids pVP212 and pVP232 were constructed with DNA fragments taken from pUC19, pMC, and JD215 (14–16). Standard DNA cloning procedures were used (17). A detailed description of the cloning steps to generate pVP212 and pVP232 is available on request.

Protocol for *lac* Repressor Protein-Mediated Purification of Proviral DNA. Genomic DNA was cleaved with restriction enzymes *Not* I/*Eco*RV or *Bam*HI. The reaction mixture was adjusted to 150 mM NaCl/10 mM EDTA/50 μg of bovine serum albumin per ml/10% (vol/vol) glycerol in a final volume of 500–1000 μl. The DNA was incubated for 20 min at room temperature with *lac* repressor fusion protein (Promega; 8 μg of *lac* repressor/400 μg of DNA). The DNA/*lac* repressor protein mixture was filtered through nitrocellulose (Gelman). The nitrocellulose was rinsed three times with 1-ml portions of wash buffer (150 mM NaCl/10 mM EDTA). The nitrocellulose was immediately placed in a 1.5-ml Eppendorf tube with forceps such that the DNA side of the filter formed the inside curvature of the rolled nitrocellulose. DNA was eluted from the nitrocellulose with 1-ml portions of elution buffer [10 mM Tris·HCl, pH 7.5/10 mM EDTA/10 mM isopropyl β-D-thiogalactoside (IPTG)] being changed every hour. The nitrocellulose was rinsed after 4 hr with 1 ml of elution buffer. All wash and rinse samples were extracted with phenol and chloroform, and DNA was precipitated with ethanol in the presence of 20 μg of yeast tRNA, which acted as a carrier. The ligated DNA was extracted with

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Abbreviations: SNV, spleen necrosis virus; HIV, human immunodeficiency virus; AMV, avian myeloblastosis virus; IPTG, isopropyl β-D-thiogalactoside; LTR, long terminal repeat.

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phenol, precipitated with ethanol, and resuspended in 10 μl of double-distilled H_2O. The ligated DNA was used to transform highly competent *Escherichia coli* cells (Stratagene or BRL). Typical efficiency with control plasmids was 5×10^8 transformants per μg of DNA. The background frequency of light blue or white colonies for bacterial cells from BRL was less than 1/20,000.

DNA Sequencing. All plasmid sequencing was performed by using the Sequenase kit and protocols (United States Biochemical), using the dGTP reactions.

Cells, Transfection, and Infection. D17 cells and .2G helper cells were grown as previously described (16). Transfections were preformed by the dimethyl sulfoxide/Polybrene procedure as described (18). Virus harvesting and virus infections were performed as described (19).

RESULTS

Protocol for the Analysis of Forward Mutations. To determine the forward mutation rate for SNV and to characterize all identified mutations at the molecular level, we constructed retroviral vector plasmids pVP212 and pVP232, which upon transfection into helper cells produce viruses VP212 and VP232, respectively. A schematic representation of an integrated provirus derived from VP212 is shown in the middle of Fig. 1. These retroviral vectors contain *neo*, the bacterial

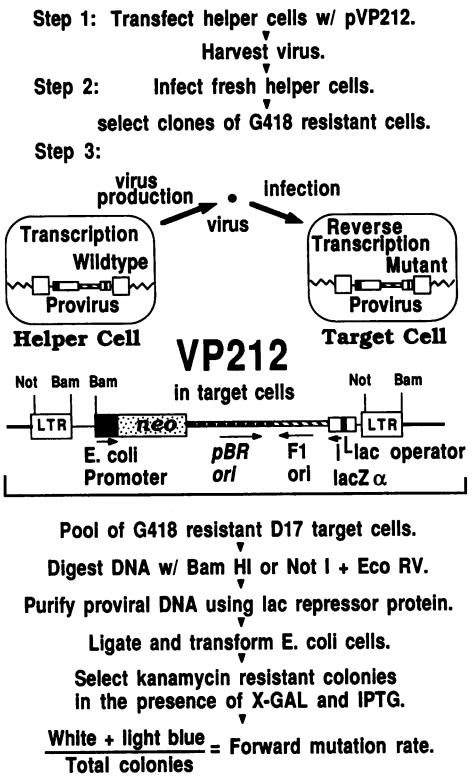


FIG. 1. Outline of experimental approach for determination of forward mutation rate for a single cycle of retroviral replication. SNV-based retroviral shuttle vectors pVP212 and pVP232 (see map of VP212 integrated provirus in the center of the figure and description in *Results*) are identical except in the orientation of the *lacZα* gene fragment. LTR, long terminal repeat. The experimental approach taken to generate pools of target cells infected with the vector viruses is also described in *Results*. The proviral DNAs were purified, ligated, and used to transform highly competent *E. coli* cells (see experimental procedures). Kanamycin-resistant bacterial colonies were selected in the presence of the 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) color indicator and the IPTG inducer. The ratio of white plus light-blue colonies to total colonies provided a forward mutation rate for a single retroviral replication cycle.

aminoglycoside 3'-phosphotransferase gene from Tn5 (20), which can be expressed in mammalian cells from the SNV LTR promoter and confers resistance to G418, a neomycin analog. The same *neo* gene can be expressed from a bacterial promoter in the vector and confers kanamycin resistance in bacterial cells. The vectors contain a bacterial origin of replication (*ori*) from pBR322 to allow replication in prokaryotic cells and an F1 origin of replication from bacteriophage M13 to facilitate isolation of single-stranded DNA for DNA sequencing. The vectors also contain the *lacZα* gene, which is expressed only in bacterial cells. It is used as a reporter of mutant proviruses arising in a single round of retroviral replication. In appropriate bacterial cells, the wild-type *lacZα* gene confers a blue color to bacterial colonies, whereas mutated *lacZα* genes give rise to white or light-blue colonies. Finally, the vectors contain the *lac* operator sequences, which bind to the *lac* repressor protein in a tight and specific manner (21).

The experimental approach is described in Fig. 1. Retroviral vector plasmids pVP212 or pVP232 were transfected into an SNV helper cell line (.2G), and G418-resistant cells were selected (step 1) (13). Virus produced from these cells was harvested and was used to infect fresh .2G helper cells. Again G418-resistant cells were selected, and individual cell clones were isolated and expanded (in the presence of anti-SNV antibodies to prevent the spread of virus during expansion) to generate several .2G helper cell clones infected with either VP212 or VP232 (step 2). Next, virus produced from the step 2 .2G helper cell clones was harvested and used to infect a permissive dog cell line, D17 (step 3). While the defective retrovirus vector virus produced from helper cell clones can readily infect D17 cells, viral structural proteins are not produced in D17 cells infected with these vectors, and virus spread does not occur. Because we used single-cell clones of helper cells (step 2), mutant viruses that may have arisen during transfection or the first round of infection did not contribute to cells that were infected during the second round (step 3).

The steps in going from a parental provirus in helper cells to a provirus in target cells are defined as a single replication cycle. These steps include transcription of proviral RNA by the cellular transcription machinery, packaging of viral RNA and release of viral particles, infection of target cells, reverse transcription of viral RNA, and integration of the newly synthesized viral DNA to generate a provirus.

We developed a procedure that uses the tight binding of the *lac* repressor protein to *lac* operator sequences to purify DNA fragments containing the *lac* operator sequence from complex mixtures of DNA fragments (see *Materials and Methods*). One proviral plasmid clone was obtained for each microgram of genomic DNA (data not shown).

Recovery and Characterization of Mutant Proviruses. Virus from five helper cell clones infected with VP212 (step 2 clone numbers 2 and 6–9) and two helper cell clones infected with VP232 (step 2 clone numbers 5 and 9) were used to harvest virus and to infect D17 cells to generate separate pools of target cells. Each pool of target cells contained from 10,000 to 100,000 different G418-resistant cells as estimated from virus titers (data not shown). A total of 16,867 kanamycin-resistant colonies were recovered. Of these, 37 colonies were either light blue or white, indicating that the *lacZα* gene was mutated at a rate of 1/456 mutants per cycle (Table 1).

All 37 clones of mutant proviruses were further characterized by plasmid DNA sequencing (Table 2 and data not shown). Several different types of mutations were found. It was also apparent that identical mutations were present in separate bacterial colonies. For example, VP212 plasmids 18, 74, and 75 all contained the same base-pair substitution. With one exception, which is discussed later, all separate plasmids containing the same mutation were isolated from the same

Table 1. Recovery of proviruses

Clone Mtn. type* (mutant)†	No. mutants/ total recovered	Mutation frequency
VP212-2	15/3621	4.1×10^{-3}
DI (3, 7, 8, 10, 12)		
D (5, 13, 21)		
D (15, 16)		
H (90, 91)		
S (97, 98)		
S (99)		
D (6)‡		
VP212-6	3/1006	3.0×10^{-3}
S (18, 74, 75)		
VP212-7	3/2843	1.1×10^{-3}
F (76)		
F (77)		
F (78)		
VP212-8	2/994	2.0×10^{-3}
F (31)		
S (35)		
VP212-9	8/3830	2.1×10^{-3}
F (40)		
DI (41)		
S (45)		
S (79, 81, 82)		
VP232-5	7/1788	3.9×10^{-3}
D (93, 95, 96, 101, 103)		
D (102)		
DI (104)		
VP232-9	0/2785	$<3.6 \times 10^{-4}$
Total	37/16,867	2.5×10^{-3}§

*Mutation types: S, base-pair substitution; F, ±1-bp frameshift; D, deletion; DI, deletions with insertions; H, hypermutation. Deletions and deletions with insertions are discussed in the following paper (22).

†Mutant isolates containing identical sequences are presented on the same line.

‡D5 (mutant no. 6) was phenotypically blue; the deletion mutation was identified through restriction mapping and DNA sequencing. Therefore, this mutation was not used in the calculation of the mutation frequencies.

§The average mutation frequency represents a weighted average with a standard error of 0.4×10^{-3} mutant per cycle.

pool of target cells, indicating that clonal amplification had occurred during expansion of target cell pools. It is also likely that up to 2-fold amplification occurred during propagation of cloned proviruses in *E. coli* prior to plating on kanamycin plates (data not shown). In any case, all proviral clones that were recovered from the same pool of target cells and contained identical mutations were probably a single mutational event rather than a mutational hotspot and are presented on the same line in the tables.

Classification of Mutations and Determination of Forward Mutation Rates. Six different base-pair substitutions (S1–S6), all transitions, were detected (Table 2). All base-pair substitutions resulted in a change in the amino acid codon within the *lacZα* open reading frame, and in two cases (S2 and S5), they resulted in an in-frame translational stop codon. S3 and S4 mutant proviruses were recovered from different pools of clones; nevertheless, they both contained the same T-to-C change, indicating that this position may be a mutational hotspot for base-pair substitutions. The overall base-pair substitution rate was 7×10^{-6} substitution per bp per cycle.

Hypermutated proviral plasmid clones (H1) exhibited 15 base-pair substitutions in a stretch of 930 base pairs. The rate of hypermutation over this stretch of nucleotides was 2×10^{-2} substitution per bp per cycle, indicating a 10,000-fold higher rate than the average substitution rate of 7×10^{-6} substitution per bp per cycle.

Table 2. Rates of substitution, hypermutation, and frameshift mutation

Mtn. type (mutant)	Nucleotide change(s)	Mtn. rate, mtn. per bp per cycle
Substitution		7×10^{-6}*
S1 (97, 98)	G to A (His to Tyr)	
S2 (99)	G to A (Gln to stop)	
S3 (18, 74, 75)	T to C (Asp to Gly)	
S4 (79, 81, 82)	T to C (Asp to Gly)	
S5 (35)	C to T (Trp to stop)	
S6 (45)	A to G (Leu to Pro)	
Hypermutation		2×10^{-2}†
H1 (90, 91)	G to A (15 subst.)	
Frameshift		1×10^{-6}‡
F1 (76)	AAAA to AAA	
F2 (77)	GGGGG to GGGG	
F3 (31)	AAA to AAAA	
F4 (40)	AAA to AAAA	
F5 (92)	AA to AAA	

*Mutation rate of substitution was calculated as follows: (11 substitutions + 2 substitutions from the hypermutated proviruses)/16,867 total proviruses recovered per 113 target nucleotides of the *lacZα* gene (6).

†Mutation rate of hypermutation was determined as follows: 15 substitutions/930 nucleotides in the hypermutated region.

‡Mutation rates of frameshifts were calculated similarly to rates of substitution, except that the target was 280 nucleotides (length of *lacZα* gene plus the *lacZα* promoter).

Five frameshift mutant proviruses (F1–F5) exhibited either +1 or −1 frameshifts in runs of identical nucleotides ranging in size from two to five nucleotides. The rate of frameshift mutation was 1×10^{-6} per bp per cycle. Analysis of the frequencies of runs of identical nucleotides and the frequencies of mutations found in the runs of each length suggested that there was a 4-fold increase in mutation frequency with each nucleotide increase in the length of the nucleotide run (data not shown).

Hypermutation in the *lacZα* Gene and in the SNV LTR U3 Regions. The two plasmid clones (90, 91) that were found to contain 15 identical base-pair substitutions over a stretch of 930 bp very likely resulted from clonal expansion of a single mutant provirus. All 15 substitutions in the hypermutant proviruses were G-to-A transitions; 8 of these substitutions preceded an A residue, and the remaining 7 were in runs of 2 to 4 G residues (Fig. 2). At two separate locations, two adjacent G residues were replaced by two A residues. One of these adjacent substitutions was located within the polypurine tract, two nucleotides 5′ of the beginning of the U3 region of the 3′ LTR.

Four substitutions in the hypermutant proviruses occurred within the open reading frame of the *lacZα* gene. Three of these resulted in a change in the amino acid encoded by the triplet (Arg to Cys, Pro to Ser, and Ser to Phe). Any one of these substitutions could have led to the light-blue colony phenotype that allowed identification of the hypermutated proviruses. The fourth substitution was a silent mutation.

The 930-bp stretch in which these substitutions occurred extended through most of the U3 region of the 3′ LTR, ending 65 bp upstream of the U3–R junction. No other mutations were found in the nucleotide sequence of the entire 4.7-kilobase (kb) hypermutant proviral plasmid (data not shown).

The two copies of the hypermutant proviruses contained 30 of the 41 substitutions identified in this population of proviruses. Two of the 13 proviruses that contained substitutions in this population were hypermutant proviruses.

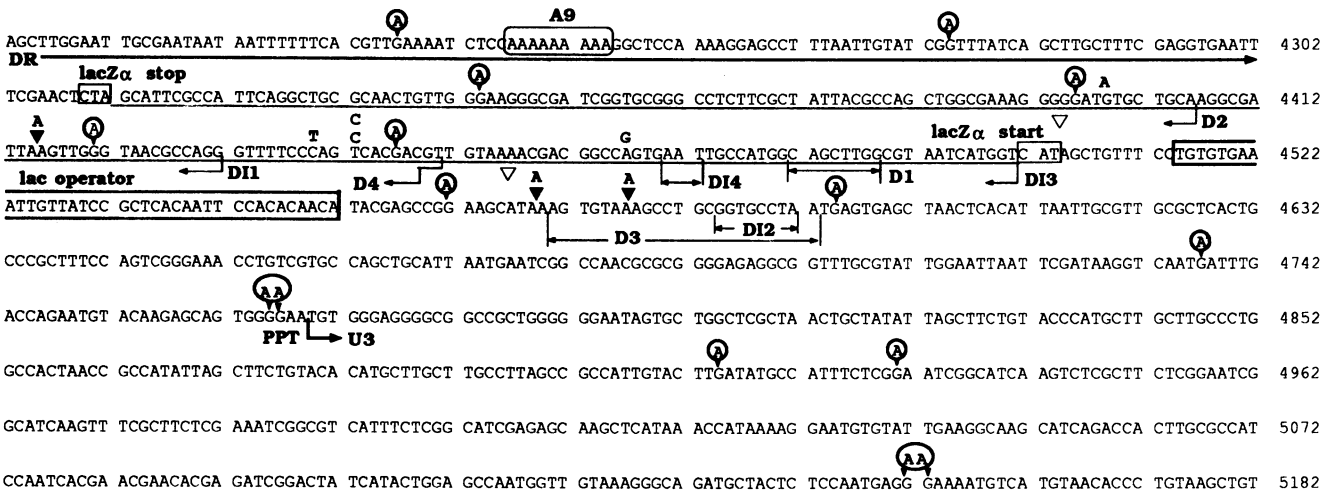


FIG. 2. Plus strand nucleotide sequence of pVP212 direct repeat, *lacZα* gene, and 3′ SNV LTR U3 region. The nucleotide sequence begins with one direct repeat of F1 sequence (shown as DR followed by a thick solid arrow below sequence). The nucleotide numbers on each line refer to numbers beginning at the end of the *Sac* I restriction site near the beginning of the 5′ LTR. The *lacZα* gene (thin line below sequence), the start and stop codons of the *lacZα* open reading frame (thin boxes), and the boundaries of the U3 sequence of SNV LTR (thin arrows below sequence) are shown. Nucleotide positions of base-pair substitutions (letters above the sequence), +1 frameshifts (letters with ▼ above the sequence), −1 frameshifts (▽ below the sequence), deletions, and deletions with insertions (solid arrows below the sequence adjacent to deletion names; D1–D5 = deletions; DI1–DI4 = deletions with insertions), and substitutions in hypermutant proviruses (circled letters above sequence) are indicated. Deletions and deletions with insertions are discussed in the following paper (22). PPT, polypurine tract; U3, unique 3′ region of SNV LTR; R, direct repeat region of SNV LTR.

Runs of Nucleotides and Direct Repeats Are Mutational Hotspots. All frameshift mutations identified involved runs of identical nucleotides, suggesting that nucleotide runs were hotspots for frameshift mutations (Table 2). We tested this hypothesis by analyzing the two A_9 and T_{10} runs (A9 and T10) present in the vectors by direct sequencing (see Fig. 3).

The A9 runs were located within a direct repeat of 110 nucleotides, constituting the transcriptional terminator of the Fd phage (Fig. 3*A*). Mutations within the direct repeats did not affect the structure or the amount of the *lacZα* peptide, and they were regarded as unselected mutations. The F1 repeats of 34 proviral clones of mutant or wild-type phenotype were sequenced. Of these, 14 clones contained only a single 110-nucleotide repeat, indicating that deletion of one direct repeat occurred in 41% of the proviral clones. It is possible that these deletions occurred as a result of template misalignments during DNA synthesis as described in the following paper (22). Three of the 14 deletion mutants containing a single direct repeat had A10 rather than the parental A9. The shift from A9 to A10 could have occurred through a frameshift event during DNA synthesis. It is also possible that the A9 to A10 shift and the deletion of one copy of the direct repeat were causally linked. The template misalignment that led to the deletion could have occurred within the A9 runs, but with a +1 misalignment, which in turn could have led to the A9 to A10 shift.

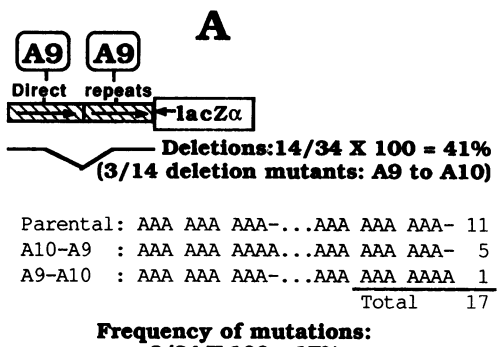
Nucleotide sequence analysis of the F1 direct repeats of the remaining 17 proviral clones which retained both copies of the direct repeat indicated that the frequency of frameshift was 17% (6/34).

The T10 runs present in the SNV LTR were also analyzed by direct sequencing (see Fig. 3*B*), and they exhibited a frequency of frameshift of 42% (16/38). This difference in the frequency of frameshifts was probably the result of the increase in the length of the runs from 9 to 10.

It should also be noted that the frameshifts in A9 differed qualitatively from the frameshifts in T10. Only +1 frameshifts were found in A9, whereas all but one frameshift event in T10 were −1 frameshifts.

DISCUSSION

We describe a system to study *in vivo* forward mutations after a single round of retroviral replication. This study was made possible by our development of a retroviral vector that can be easily recovered in large numbers after integration into host genomic DNA. This method allowed us to recover and analyze several mutations in the *lacZα* gene. The method is technically simple in comparison to standard methods used for cloning of single-copy sequences from complex genomes, and it can be used in any experiment in which DNA is



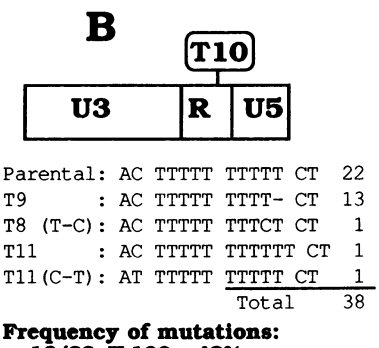


FIG. 3. Frameshift mutations in A9 and T10 runs. (*A*) Nucleotide runs of 9 A residues (A9 in rounded boxes) within direct repeats of 110 nucleotides (hatched boxes with arrows). The orientation and location of the *lacZα* gene (open box) is also shown. Bent line below the direct repeats indicates deletion of one copy of the direct repeat. Also shown are nucleotide sequences of runs in 17 clones that retained both copies of the direct repeats. A hyphen indicates wild-type nucleotide. (*B*) Nucleotide run of 10 T residues (T10 in rounded boxes) within the R region of SNV LTR (open boxes) is shown. The sequences obtained for 38 clones are shown below. The hyphen indicates deletion of a nucleotide.

introduced into eukaryotic cells and is retrieved, provided that the DNA can be tagged with the *lac* operator sequence.

Substitutions. Even with the small number of substitutions that we characterized, transitions predominated over transversions. We also identified a potential hotspot for substitutions, since the same substitution was recovered from two different pools of step 3 clones. The substitution mutations could not be explained by the misalignment mechanism previously proposed (23). The substitution mutations we found were in different locations than those found for human immunodeficiency virus (HIV) and avian myeloblastosis virus (AMV) in *in vitro* assays (6).

Hypermutations. We postulate that the hypermutations occurred through the action of an error-prone reverse transcriptase or RNA polymerase II. The error-prone polymerase could arise through errors occurring during transcription or translation. Recently, the combined error rate of transcription and translation was determined as 10^{-4}/bp by study of reversion of mutations introduced in the luciferase gene (S. Yang, personal communication). Since we recovered 1.7×10^4 proviruses, it is possible that a small number of virions that formed these proviruses contained mutant reverse transcriptases. The number of reverse transcriptase molecules present in each virion has been estimated to be 40–110 (24, 25). It is not known how many reverse transcriptase molecules contribute to the synthesis of each DNA molecule that integrates to become a provirus. The 15 G-to-A substitutions found in a patch of 930 nucleotides probably occurred during a single polymerization step either during minus strand or during plus strand DNA synthesis. It is possible that this particular region of the provirus was synthesized by a mutated, error-prone, reverse transcriptase molecule, and that the rest of the provirus was synthesized by wild-type reverse transcriptase molecules. Alternatively, this provirus could have resulted from recombination between a DNA molecule that was completely replicated by an error-prone polymerase and one that was synthesized by a wild-type polymerase (19).

Almost 75% of the base pair substitutions that we found were present in the hypermutant proviruses. The rate of recombination in retroviral replication was recently determined as 4%/kbp per cycle (19). With this high recombination rate, substitutions in hypermutant proviruses could quickly spread throughout the viral population. This hypothesis raises the possibility that many of the substitution mutations in viral populations result from hypermutant proviruses and recombination.

Deletions of Direct Repeats and Frameshifts. Our results confirm and quantify previous observations that direct repeats in retroviral vectors are deleted at a high frequency (26, 27). Our results indicate that runs of identical nucleotides constitute hotspots for frameshift mutations. The frameshift mutations could have occurred by the Streisinger strand-slippage mechanism (28).

Kunkel and coworkers (6) found that HIV reverse transcriptase was much more prone to cause frameshift mutations in runs than the AMV reverse transcriptase during *in vitro* DNA-dependent DNA synthesis. The SNV reverse transcriptase appears to be more like the HIV enzyme than the AMV enzyme in its tendency to generate frameshift mutations.

We found that the A9 run that is a part of a direct repeat gave rise to a high frequency of +1 frameshifts, whereas a T10 repeat in the SNV LTR R region primarily gave rise to −1 frameshifts. It is possible that the sequence context, which has been shown to be important in the mutation frequency in different runs, is involved (6). It is also possible that the fact that the A9 is within a direct repeat played a role in causing the +1 frameshifts.

Two transition mutations were found in T10. These transition mutations could have occurred by the previously proposed mechanism of template misalignment (23). Assuming that misalignments were involved, the T8 (T-to-C) transition mutation occurred during plus-strand DNA synthesis, creating a CTCT direct repeat at the end of a T8 run. Similarly, the T11 (C-to-T) transition mutation occurred during minus-strand DNA synthesis, creating a C-to-T change at the beginning of the T10 run.

Another possibility is that the T10 run had a higher frequency of mutations because it was involved in the first strand transfer, which is essential for retroviral replication, and because misalignments during the strand transfer events lead to a greater frequency of mutations. This possibility would require that first strand transfer occurred before synthesis of the 5′ R region was completed.

The results presented here and the results of others (29) suggest that reverse transcriptase is an enzyme with low processivity; the affinity of the enzyme for the template may be low in comparison to other DNA polymerases. This property is perhaps necessary for an enzyme that participates in the strand transfer steps essential for viral replication.

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1. Rous, P. & Murphy, A. B. (1914) *J. Exp. Med.* **19**, 52–69.
2. Temin, H. M. (1960) *Virology* **10**, 182–197.
3. Temin, H. M. (1961) *Virology* **13**, 158–163.
4. Gojobori, T. & Yokoyama, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4198–4201.
5. Drake, J. W., Allen, E. F., Forsberg, S. A., Preparata, R. M. & Greening, E. O. (1969) *Nature (London)* **221**, 1128–1132.
6. Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H. & Kunkel, T. A. (1989) *J. Biol. Chem.* **264**, 16948–16956.
7. Preston, B. D., Poiesz, B. J. & Loeb, L. A. (1988) *Science* **242**, 1168–1171.
8. Roberts, J. D., Bebenek, K. & Kunkel, T. A. (1988) *Science* **242**, 1171–1173.
9. Roberts, J. D., Preston, B. D., Johnston, L. A., Soni, A., Loeb, L. A. & Kunkel, T. A. (1989) *Mol. Cell. Biol.* **9**, 469–476.
10. Ricchetti, M. & Buc, H. (1990) *EMBO J.* **9**, 1583–1593.
11. Coffin, J. M., Tsichlis, P. N., Barker, C. S. & Voynow, S. (1980) *Ann. N.Y. Acad. Sci.* **354**, 410–425.
12. Leider, J. M., Palese, P. & Smith, F. I. (1988) *J. Virol.* **62**, 3084–3091.
13. Dougherty, J. P. & Temin, H. M. (1988) *J. Virol.* **62**, 2817–2822.
14. Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33**, 103–119.
15. Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M. & Fritz, H.-J. (1989) *Nucleic Acids Res.* **17**, 4441–4454.
16. Dougherty, J. P. & Temin, H. M. (1986) *Mol. Cell. Biol.* **6**, 4387–4395.
17. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
18. Kawai, S. & Nishizawa, M. (1984) *Mol. Cell. Biol.* **4**, 1172–1174.
19. Hu, W.-S. & Temin, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1556–1560.
20. Jorgensen, R. A., Rothstein, S. J. & Reznikoff, W. J. (1979) *Mol. Gen. Genet.* **117**, 65–72.
21. Mossing, M. C. & Record, M. T., Jr. (1985) *J. Mol. Biol.* **186**, 295–305.
22. Pathak, V. K. & Temin, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6024–6028.
23. Kunkel, T. A. & Alexander, P. S. (1986) *J. Biol. Chem.* **261**, 160–166.
24. Panet, A. & Kra-Oz, Z. (1978) *Virology* **89**, 95–101.
25. Bauer, G. & Temin, H. M. (1980) *J. Virol.* **33**, 1046–1057.
26. Xu, H. & Boeke, J. D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8553–8557.
27. Rhode, B. W., Emerman, M. & Temin, H. M. (1987) *J. Virol.* **61**, 925–927.
28. Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E. & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol.* **31**, 77–84.
29. Majumdar, C., Abbotts, J., Broder, S. & Wilson, S. H. (1988) *J. Biol. Chem.* **263**, 15657–15665.